Localization of Nox2 N-terminus using polyclonal antipeptide antibodies

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Nox2/gp91^{phox} (where phox is **ph**agocyte **ox**idase) is the catalytic membrane subunit of the granulocyte NADPH oxidase complex involved in host defence. The current model of membrane topology of Nox2 is based upon the identification of glycosylation sites, of regions that interact with the regulatory cytosolic factors and of the epitopes recognized by antibodies. So far, the localization of the N-terminus of Nox2 was only speculative. In order to clarify this localization, we raised a polyclonal antiserum against the N-terminal sequence M^{1}GNWVAVNEGL^{11}. Purified antibodies recognize the mature protein as a broad band at 91 kDa (glycosylated form) or a band at 55 kDa after deglycosylation. Immunocytochemistry and flow-cytometry analysis show a strong binding of the anti-N-terminal antibodies to differentiated HL60 cells and neutrophils respectively, after permeabilization only. The N-terminus of Nox2 is therefore present in the mature protein and is located to the cytoplasmic side of the plasma membrane.

Key words: anti-peptide antibody, confocal microscopy, flow cytometry, gp91^{phox}/Nox2, membrane topology, NADPH oxidase.

INTRODUCTION

Nox2/gp91^{phox} (where phox is phagocyte oxidase) is the catalytic membrane subunit of the NADPH oxidase, an enzyme complex found in phagocytic leucocytes which catalyses the formation of superoxide anion ($O_2^{\bullet -}$), a reactive oxygen species involved in the host defence against pathogens. It is an integral membrane protein of 570 amino acids, which is predicted to contain multiple transmembrane domains [1,2]. The N-terminal part of the protein was shown to be involved in haem binding through histidine residues 101, 115, 209 and 222, located in helices III (101 and 115) and V (209 and 222) [3]. After proteolytic treatment of partially purified cytochrome b_{558}, a spectrally stable fragment comprising the whole N-terminus and ending at amino acids 320 or 363 was recovered [4]. gp91^{phox} has been proposed to contain the binding sites for the NADPH oxidase prosthetic group, FAD, and the substrate, NADPH [5,6]. Based on a weak primary structure similarity between the C-terminal domain of gp91^{phox} and ferredoxin–NADP$^+$ reductase (FNR), a structural model for the nucleotide-binding region of the gp91^{phox} C-terminus was predicted based on the known structure of FNR [7]. As FNR is a soluble protein and the C-terminal region contains the predicted substrate-binding pocket, it is proposed that the C-terminal half of gp91^{phox} is soluble and has a cytoplasmic location. Human gp91^{phox} is a highly glycosylated protein and appears on Western blots as a broad smear from 120 kDa to 60 kDa, average approx. 91 kDa. The glycosylation has previously been demonstrated to be N-linked [8] and to be attached to residues Asn^{131} and Asn^{148} located in the loop between predicted transmembrane helices III and IV (numbering of the helices according to the main current model, Figure 1), and Asn^{239} between transmembrane helices V and VI of gp91^{phox} [9]. Therefore the current models for the membrane topology of gp91^{phox} place these three glycosylation sites to the exterior of the membrane. Monoclonal antibody (mAb) 7D5 [10,11] and polyclonal anti-L$_{123}$ (S^{151}YLNFARKRIKN-PEGGLYLAVTL^{173}) antibody [12] have both been reported to bind to intact neutrophils, demonstrating that their epitopes are exposed to the exterior of the cell. On the other hand, mAbs 54.1 [13], NL7 [14] and polyclonal anti-L$_c$ (I^{551}SNSESG-PRGVHFIFNKENF^{570}) antibody [12] can only bind following permeabilization of the membrane, therefore placing their individual epitopes on the cytosolic side of the membrane. The use of phage-display libraries has mapped the epitopes for these mAbs to: I^{160}KNP^{163} plus R^{226}IVRG^{230} for 7D5 [11], confirming the extracellular location of region 151–173, P^{383}KIAVDGP^{390} for mAb 54.1 [13], E^{498}KDVITGL^{505} for mAb 48 [15] and E^{498}KDVI-TGLK^{506} for NL7 [14]. The intracellular location of the epitopes for mAb 54.1, mAb 48, mAb NL7 and anti-Lc antibody provides supporting evidence that the C-terminal domain of gp91^{phox} protrudes into the cytoplasm of the cell. After activation, p47^{phox}, p67^{phox} and Rac, the cytosolic subunits of the NADPH oxidase, interact with the membrane components to form a functional enzyme complex. Using phage-display libraries, three regions of gp91^{phox} which bind p47^{phox} were identified as S^{86}TRVRRQL^{93}, F^{450}EWFADLL^{457} and E^{554}SGPRGVHFIF^{564} [16]. Residues 86–93 are between predicted transmembrane domain I and II and contain a high density of positively charged residues. Arg^{91} and Arg^{92} from this region were shown to be critical for the activity of cytochrome b_{558} [17], presumably by interaction with p47^{phox}. For residues 450–457, their involvement in p47^{phox} binding is currently questioned by the data obtained with mAb NL7 [14]. Recently, a number of homologues of gp91^{phox}, named Nox, have been identified in the human [18], *Drosophila*, *Caenorhabditis elegans* (reviewed in [19]), *Dictyostelium* (B. Lardy, M. Bof, L. Aubry, M. H. Paclet, F. Morel, M. Satre and G. Klein, unpublished work), *Arabidopsis* [20] and other plant [21,22] genomes. Although the amino acid sequences of the human homologues show varying degrees of identity [18], they all exhibit a similar hydropathy plot. An alternative to the main current model has recently been proposed by Cheng et al. [18] based on the suggestion that the N-terminal 30–35 amino acids of Nox1, Nox2, Nox3 and Nox4 act as cleavable signal peptides. In the case of gp91^{phox}/Nox2, the

Abbreviations used: ECL, enhanced chemiluminescence; FNR, ferredoxin–NADP$^+$ reductase; mAb, monoclonal antibody; Nox, **N**ADPH **ox**idase; phox, **ph**agocyte **ox**idase.

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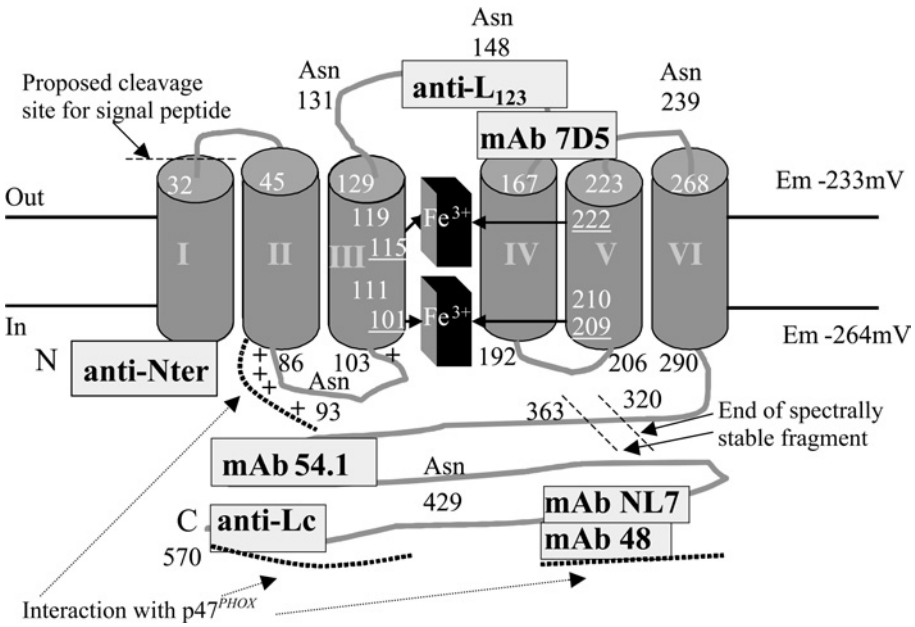

Figure 1 Predicted membrane topology of Nox2

The diagram represents the current knowledge regarding the membrane topology of Nox2/gp91^{phox}. Predicted transmembrane helices are numbered according to the current models. Asn^{131}, Asn^{148} and Asn^{239} are the sites of attachment of the N-linked glycosylation. Asn^{93} and Asn^{429} are not glycosylated. Histidine residues from helices III and V are labelled and those directly involved in haem binding [3] are underlined. The boxed texts are the identified epitopes for antibodies, of which only anti-L_{123} antibody and 7D5 are accessible in the intact cell. The sites of sequence similarity to the peptides identified from a phage display library as interacting with p47^{phox} are shown as broken lines. The polybasic region 86–103 is indicated by +. The topology of the N-terminus had not previously been investigated and is established by the present study. There is current controversy regarding the actual nature of the N-terminal amino acids in the mature protein. Cheng et al. [18] have proposed that the N-terminal is a signal sequence the cleavage site for which is indicated by the arrow. Two dotted lines indicate two possible ends of a spectrally stable N-terminal fragment [4]. Em represents the membrane potential.

predicted signal peptide would include the 30 amino acids which form the first predicted transmembrane domain. This hypothesis is in direct conflict with the N-terminal sequence data of gp91^{phox} that was originally determined from the protein purified from granulocytes [23]. The amino acid sequence clarified the position of the initiating AUG codon within the identified gene [1,24]. Therefore most models predict that the N-terminal amino acids of gp91^{phox} would protrude on the cytosolic side of the membrane, as shown in Figure 1, but they are not supported by experimental evidence.

To clearly identify the localization of the N-terminus of the protein, we raised an antiserum against the N-terminal sequence of the protein M^{1}GNWAVNEGL^{10}-Y (with a tyrosine residue added to the C-terminal end as indicated). As a control we used an antibody directed against the peptide L^{153}NFARKRIKNPEGGLY^{168} that had previously been shown to be extracellular [12]. By performing Western blotting, flow cytometry analysis and confocal microscopy with these antibodies, we clearly demonstrated that the N-terminus part of Nox2 is present in the mature neutrophil protein and that it is localized in the cytoplasm. Our study provides the first experimental evidence for the intracellular location of the N-terminus of Nox2.

EXPERIMENTAL

Materials

The following materials were supplied by the indicated companies: N-glycosidase F (recombinant, *Escherichia coli*), *n*-octyl glucoside (Roche diagnostics, Meylan, France); ECL® (enhanced chemiluminescence) Western blotting detection reagents (Amersham Biosciences, Orsay, France); anti-rabbit IgG (whole molecule) FITC-conjugate (Sigma-Aldrich, Saint-Quentin-Fallavier, France); Cy™2-labelled anti-rabbit polyclonal antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.); peptides (Immunograde, 80 % pure) were obtained from Neosystem (Strasbourg, France). RPMI medium was from Invitrogen (Paisley, Renfrewshire, Scotland, U.K.), Inject ovalbumin from Pierce (Perbio, Brebieres, France), Affi-Gel 10 from Bio-Rad (Marnes-la-Coquette, France), Centricon 30 from Millipore (Saint-Quentin-en-Yvelines, France). All other chemicals were from Sigma.

Production of antipeptide antibodies

For the N-terminal peptide (M^{1}GNWAVNEGLS^{11}-Y) (P_{Nter}), a tyrosine residue was added C-terminally to the sequence of the peptide to allow coupling to the carrier protein. The second peptide ($P_{153–168}$) corresponded to residues L^{153}NFARKRIKNPEGGLY^{168} with the tyrosine present in the sequence itself. After protection of lysine residues, peptides were coupled to ovalbumin as carrier protein, as described previously [25], using bis-diazobenzidine. The peptide–ovalbumin (coupled peptide) was used for immunization. Pre-immune serum was taken and five injections of 0.5 ml of coupled peptide were made at 3 week intervals. The rabbits were bled 10 to 15 days after the fifth injection.

Affinity purification of antibodies

A peptide–L-tyrosine resin was made in two steps. First, L-tyrosine was covalently attached to an activated medium (Affi-Gel 10) and residual binding sites were saturated by 1-h incubation with 300 mM ethanolamine**.** The peptide (2 μmol for 5 ml of resin) was then added and covalently bound to the L-tyrosine resin using bis-diazobenzidine [25] as above. Approx. 10 ml of serum was

incubated with the peptide–L-tyrosine resin (5 ml of dry resin) for 1 h at 4 °C with gentle shaking. The resin was washed first at high ionic strength, i.e. 50 mM Tris/HCl (pH 8.0)/500 mM NaCl, then twice with PBS (pH 7.4; 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4). For elution, the resin was poured into a column, washed with PBS and elution was performed by 5 ml of 0.1 M glycine/HCl, pH 1.8, buffer (pH adjusted at 25 °C). The pH was immediately readjusted in eluted fractions with 0.1 volume of 1 M Trizma base. Elution was followed by monitoring the A_{280}. The fractions containing proteins (immunoglobulins) were pooled, and the pH was adjusted to neutral. Antibodies were concentrated on a Centricon 30 device and the buffer was exchanged for PBS. Purified antibodies were supplemented with 50 % glycerol and stored at − 20 °C until use.

Gel electrophoresis

Cytochrome b_{558} purified from neutrophils (50 pmol) was submitted to SDS/10 %-(w/v)-PAGE. Proteins were detected by staining with silver nitrate [26].

Western blotting

The membrane fraction was isolated from human neutrophils and cytochrome b_{558} was purified as previously described [27]. Crude membrane (100 μg) or purified cytochrome b_{558} (8 pmol) was subjected to SDS/PAGE (10 % gel) and submitted to Western blotting and probed with 1:1000 dilution of the antibody. The binding of the first antibody was detected following the addition of horseradish peroxidase-conjugated anti-rabbit secondary antibody, using the ECL® detection kit. A control sample was incubated in parallel with pre-immune serum. In some experiments, antibodies were pre-incubated with the peptide used for immunization (50 μg/ml) for 1 h prior to probing the nitrocellulose membrane.

Deglycosylation experiments

Purified cytochrome b_{558} (10 pmol) was denatured by heating at 100 °C for 2 min in presence of 0.2 % (w/v) SDS and 1 % (v/v) β-mercaptoethanol. At the end of the incubation, 34 mM *n*-octyl glucoside and 2 units of N-glycosidase F were added for an overnight incubation at 37 °C [28,29].

Culture and differentiation of HL60 cells

HL60 cells, a human promyelocytic cell line [30], were maintained in RPMI 1640 supplemented with 10 % (v/v) foetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin, at 37 °C, in the presence of 5 % CO_2. The cultures of HL60 cell were initiated at 5×10^5 cells/ml on a weekly basis. Differentiation of HL60 cells was induced by the addition of 1.25 % (v/v) DMSO to cells at 5×10^5 cell/ml. The cells were harvested by centrifugation at 800 ***g*** for 10 min, on the fifth day following the addition of DMSO [31].

Immunocytochemistry on HL60 cells by confocal microscopy

Differentiated and undifferentiated HL60 cells were harvested by centrifugation at 800 ***g*** for 10 min. The cells were washed twice in 10 ml PBS, pelleted at 600 ***g*** for 10 min, prior to being resuspended in 0.5 ml of PBS. The cells were allowed to settle on round glass coverslips for 10 min at 25 °C and washed twice for 10 min with PBS. Cells for permeabilization were fixed with 4 % (v/v) formaldehyde in PBS for 10 min immediately followed by permeabilization with 0.2 % (v/v) Triton X-100 in PBS for 2 min. Non-permeabilized cells were washed twice with PBS. Both Triton-X-100-treated and non-treated cells were incubated (three 10 min incubations) with 0.2 % (w/v) BSA in PBS (BSA/PBS) prior to 1-h incubation with primary antibody diluted 1:500 in BSA/PBS. The cells were washed (three 10 min washes) with BSA/PBS prior to incubation with Cy™2-labelled anti-rabbit secondary antibody for 1 h and subsequent washes. The location and intensity of the Cy™2 fluorescence were recorded on an inverted Bio-Rad MRC 600 Confocal microscope as described previously [31].

Superoxide assay

The differentiation of HL60 cells was confirmed by their ability to generate superoxide in response to activators of the NADPH oxidase [31]. The release of superoxide was measured continuously as the reduction of cytochrome *c* (550–540 nm) at 37 °C, using a double-beam spectrophotometer. The NADPH oxidase was activated by the addition of 50 nM PMA and inhibited by either 10 μM diphenylene iodonium or 50 μg/ml superoxide dismutase.

Flow cytometry

Human neutrophils were isolated from citrated venous blood of healthy volunteers using a 33 % (v/v) Hypaque–Ficoll gradient. After 20 min centrifugation at 800 ***g*** at 20 °C, the pellet was submitted to an hypotonic lysis for 5 to 15 min on ice [27]. After 5 min centrifugation at 350 ***g*** at 4 °C, the neutrophil pellet was collected and washed once in PBS. Neutrophils were suspended in PBS/BSA/$CaCl_2$ [PBS containing 0.2 % (w/v) BSA and 0.5 mM $CaCl_2$] at the concentration of 10^7 cells/ml, and fixed on ice by addition of an equal volume of 2 % (w/v) paraformaldehyde. After fixation (15 min), cells were centrifuged, washed once in PBS/BSA/$CaCl_2$ and resuspended in saponin buffer [PBS/BSA/$CaCl_2$ containing 0.01 % (w/v) saponin] at the concentration of 10^7 cells/ml for a 10-min incubation on ice. After permeabilization, cells (5×10^5) were incubated on ice for 30 min with 100 μl of primary rabbit antibody (50 μg/ml of non-immune IgG or anti-peptide IgG in saponin buffer), then washed twice with 500 μl of saponin buffer, and resuspended in 150 μl of FITC-conjugated goat anti-rabbit antibody, diluted 1:200 in saponin buffer. After 30 min incubation on ice, cells were washed twice with 500 μl of saponin buffer before being resuspended in 500 μl of PBS/BSA/$CaCl_2$. Binding of antibodies to intact cells was performed as described above without treatment with saponin buffer. Fluorescence intensity (FL1) of the FITC-labelled neutrophils was measured on a FACScalibur (Becton Dickinson) cytometer.

RESULTS

Immunodetection of gp91^{phox} by Western blot

Although most models place the N-terminus of Nox2 on the cytoplasmic face of the plasma membrane, experimental evidence for this localization was missing. An antiserum was therefore raised to the synthetic peptide M^1GNWAVNEGLS^{12}-Y (P_{Nter}), the first 11 amino acids encoded by the Nox2 cDNA. The peptide L^{153}NFARKRIKNPEGGLY^{168} ($P_{153–168}$), part of the L_{123} peptide shown to have an extracellular localization [12], was used for immunization under the same conditions.

On a Western blot, the antibody directed against the N-terminal peptide (anti-P_{Nter}) labelled specifically only one broad band at approx. 91 kDa in granulocyte membranes (Figure 2A). This band was also detected by the antibody directed against the peptide corresponding to amino acids 153–168 of gp91^{phox} (anti-$P_{153–168}$)

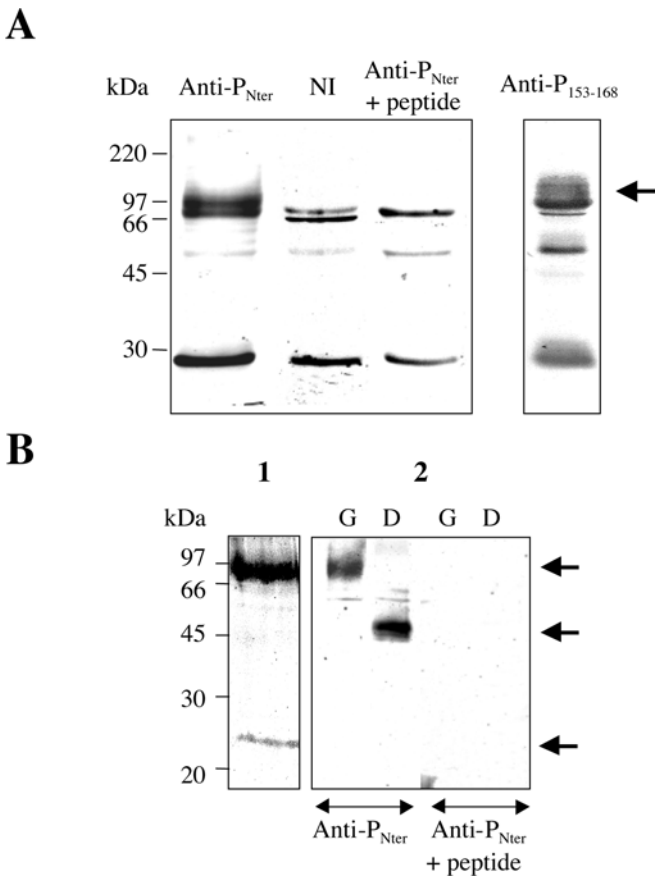

Figure 2 Western blot analysis of neutrophil fractions with the antibody against Nox2 N-terminus

Membrane protein or purified cytochrome b_{558} were separated on a SDS gel. (**A**) Western blot of a membrane fraction from human neutrophils (100 μg/lane) analysed with the anti-(Nox2 N-terminus) antibody (anti-P_{Nter}), with the non-immune antibody (NI) or with the anti-P_{Nter} pre-incubated with the peptide used for immunization (50 μg oF peptide/ml of anti-P_{Nter}, 1/1000 solution) for 1 h prior to probing the nitrocellulose membrane. A control was revealed with the anti-$P_{153-168}$, 1/1000. The immune complexes were detected by ECL®. (**B**) SDS/PAGE and Western blot of purified cytochrome b_{558}. Purified cytochrome b_{558} (50 pmol) was submitted to SDS/PAGE (10 %) and silver stained (panel 1). Western blot was performed on purified cytochrome b_{558} (8 pmol) glycosylated (G) or treated with N-glycosidase F (D) using anti-P_{Nter} (1/1000) or anti-P_{Nter} pre-incubated with the peptide used for immunization [as described in (**A**)] (panel 2). The immune complexes were detected by ECL®.

(Figure 2A), but was absent from the blots probed with non-immune immunoglobulins or pre-incubated with the competitor peptide (Figure 2A). To confirm that the band detected at 91 kDa was gp91^{phox}, a Western blot was performed on cytochrome b_{558} purified from neutrophils. The purity of the fraction was analysed by SDS/PAGE (Figure 2B, lane 1) and by its specific activity (15–20 nmol of haem *b*/mg of protein). Both p22^{phox} and gp91^{phox} were present in this fraction (Figure 2B, lane 1), but only gp91^{phox} was detected by the anti-P_{Nter}, as shown by the single broad band at 91 kDa that disappeared in presence of the competitor peptide (Figure 2B, lanes G, anti-P_{Nter} versus anti-P_{Nter} + peptide). A non-specific band at 28 kDa in the membrane fraction was not p22^{phox}, since it was not labelled when purified cytochrome b_{558} was loaded on to the gel.

gp91^{phox} is a highly glycosylated protein which travels on a gel as a broad band with average molecular mass of 91 kDa. The treatment of purified cytochrome b_{558} with N-glycosidase F led to a specific decrease in the molecular mass of gp91^{phox} (from 91 kDa to 55 kDa), as reported previously [28,29]. After deglycosylation, the binding of anti-P_{Nter} to purified cytochrome b_{558} was not affected, and the specific labelled band was at 55 kDa, as expected for the deglycosylated form of gp91^{phox} (Figure 2B, lanes D, anti-P_{Nter} versus anti-P_{Nter} + peptide). The detection of gp91^{phox} by the antibody in both the isolated granulocyte membrane fraction and the purified cytochrome b_{558} demonstrates that the epitope is present in the mature protein and is not cleaved as the result of the post-translational processing. Therefore the first 30 amino acids of Nox2 are not a cleavable signal peptide.

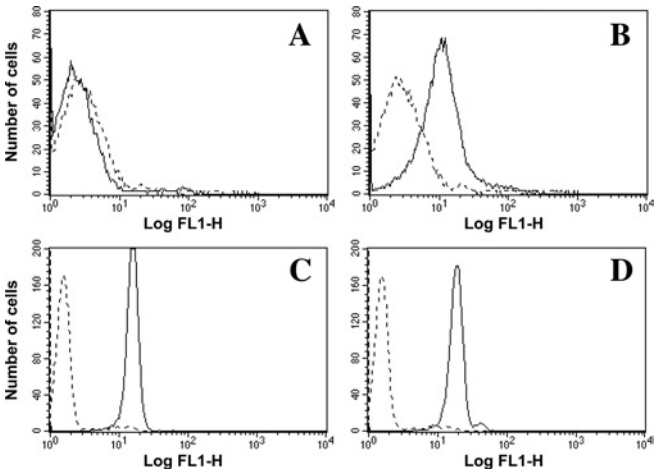

Figure 3 Flow-cytometry analysis of cytochrome b_{558} in human neutrophils

Human neutrophils (5×10^5 cells) were fixed with 1 % (w/v) paraformaldehyde and labelled with antibodies immediately (**A**, **B**) or after saponin-permeabilization (**C**, **D**) as described in the Experimental section. The antibodies used were non-immune immunoglobulins (broken line), anti-P_{Nter} (**A**, **C**; continuous line) or anti-$P_{153-168}$ (**B**, **D**; continuous line). The antibody-labelled cells were stained with FITC-conjugated secondary antibody, and the fluorescence was measured.

Immunodetection of gp91^{phox} on human neutrophils by flow cytometry

To check the localization of the N-terminal peptide of gp91^{phox}, we performed flow cytometry on human neutrophils using affinity-purified antibodies to the N-terminal peptide (anti-P_{Nter}) and to the 153–168 sequence (anti-$P_{153-168}$). A negative control with non-immune antibodies gave only a background labelling.

As shown in Figure 3(A), anti-P_{Nter} did not stain the cells extracellularly. The signal was similar to that obtained with non-specific antibody (Figure 3A, continuous line versus broken line). After permeabilization by saponin, a clear label was observed (Figure 3C, continuous line versus broken line). So far, only two antibodies, mAb 7D5 and L_{123}, have been shown to recognize an extracellular epitope. We therefore used the polyclonal antibody anti-$P_{153-168}$ that recognizes part of L_{123} peptide as a control for an extracellular localization. Binding of anti-$P_{153-168}$ was clearly above the background for intact cells, compared with anti-P_{Nter} (Figure 3B versus 3A), but labelling was slightly increased when neutrophils were permeabilized by saponin (Figure 3D). The fluorescence increase in permeabilized neutrophils could be explained by the binding of anti-$P_{153-168}$ to the cytochrome b_{558} molecules inserted in the granule membranes, molecules that were not available in intact cells.

These experiments clearly show that the N-terminus of gp91^{phox} is not accessible to antibodies when neutrophils are intact and that it becomes available when they are permeabilized.

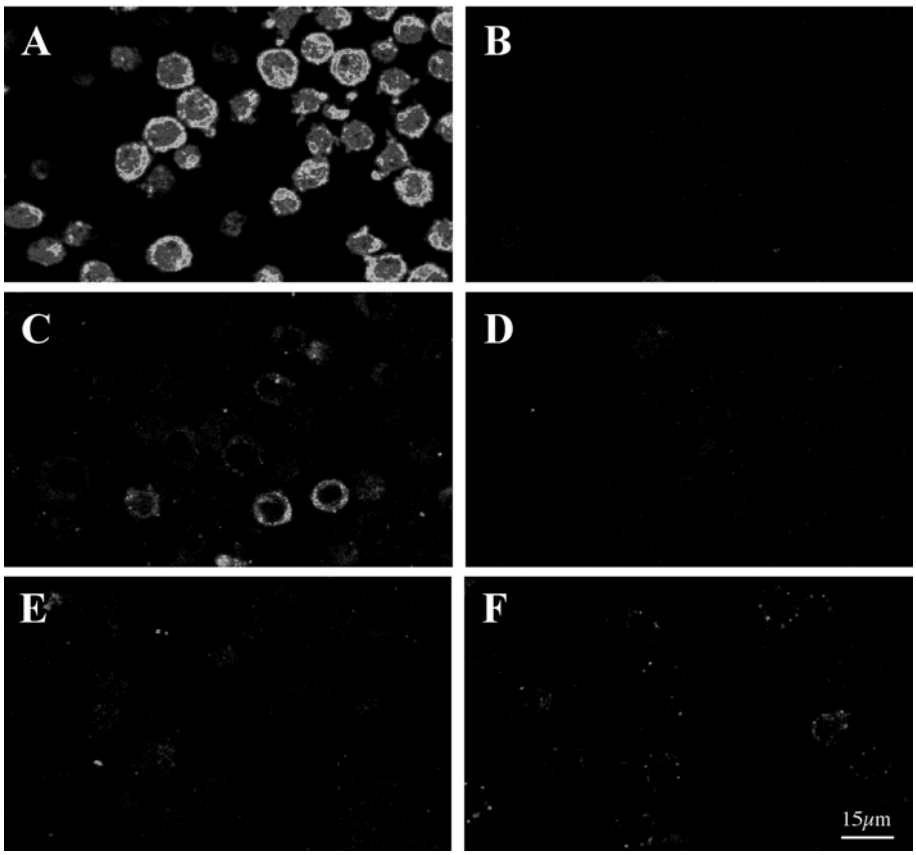

Figure 4 Immunocytochemistry of permeabilized and non-permeabilized HL60 cells

HL60 cells were differentiated, permeabilized and immunostained with anti-P_{Nter} (**A**–**D**) or non-immune serum (**E**, **F**) as described in the Experimental section. The images shown in the Figure are a Kalman average of 5 consecutively collected scans of differentiated HL60 (**A**, **B**, **E**, **F**) and undifferentiated HL60 (**C**, **D**) cells, which had been permeabilized with Triton X-100 (**A**, **C**, **E**) or left untreated (**B**, **D**, **F**). The scale bar in (**F**) represents 15 μm and is applicable to all 6 panels (**A**–**F**).

Immunodetection of gp91^{phox} on differentiated HL60 cells by confocal microscopy

The incubation of the promyelocytic HL60 cells with DMSO for 5–7 days induces them to differentiate into neutrophil-like cells. The process is associated with an increase in the ability of these cells to generate superoxide in response to an oxidase activator [31] and with an increase in expression of the individual subunits of the NADPH oxidase, including gp91^{phox} (Nox2) [32].

To determine the cellular location of the N-terminal part of Nox2, we incubated both Triton X-100 treated and non-treated HL60 cells with the N-terminal Nox2 polyclonal antibody (Figure 4). The binding of the antibody and its cellular location were determined using a fluorescently labelled secondary antibody and a confocal microscope.

A strong immunofluorescence was observed with differentiated HL60 cells which had been permeabilized with Triton X-100, incubated with anti-P_{Nter} (Figure 4A). A much lower intensity of emitted fluorescence was observed from differentiated HL60 cells which had not been treated with Triton X-100 (Figure 4B), and corresponded to the low non-specific binding measured in presence of pre-immune serum in both permeabilized and intact cells (Figures 4E and 4F respectively). In undifferentiated HL60 cells, a low but specific labelling was observed after permeabilization, indicating that a small amount of cytochrome b_{558} was already present in the membrane of these cells (Figure 4C versus 4D). As predicted, gp91^{phox} antigen shows increased expression upon differentiation of HL60 cells into neutrophil-like cells. In the absence of the anti-P_{Nter} antibody, no immunostaining was observed for differentiated HL60 cells either with or without permeabilization (results not shown), indicating that the immunostaining observed in Figure 4(A) is not due to cross-reactivity of the secondary antibody with the HL60 cells.

The failure of the antibody to detect its antigen in the absence of an agent which disrupted the integrity of the plasma membrane confirms that the N-terminal of Nox2 protrudes on the cytosolic face of the plasma membrane. We therefore conclude that the N-terminus has an intracellular location. The membrane topology of gp91^{phox}, including all previously published experimental data (see the Figure for details and references) and the present study, is illustrated in Figure 1.

DISCUSSION

In the absence of crystallographic studies, topography analysis of membrane proteins relies on several approaches. In the case of gp91^{phox}/Nox2, the number of predicted transmembrane regions ranges from 5 to 8. Many immunological approaches were undertaken, but most antibodies map to the C-terminal region and to the external loop that contains glycosylation sites. Data have accumulated that the C-terminal region of the protein is soluble. It has a cytoplasmic location and it is the site of binding for FAD, NADPH and the cytosolic factors, in particular p47^{phox}. Another 'hotspot' for antibody recognition is the extracellular region comprising two loops and three effective glycosylation

sites. There were no experimental data to confirm whether the N-terminal region could be placed either on the cytoplasmic or on the external side of the plasma membrane. The lack of information about this region could be explained by the small size, the poor solubility and immunogenicity of this part of the protein. In the present paper, we used for the first time a polyclonal antibody directed against the N-terminal peptide of gp91^{phox} to clearly identify the localization of this region.

The N-terminal 31 amino acids of Nox2 are present in the mature protein and do not act as a cleavable signal peptide as proposed by Cheng et al. [18]. This result is in agreement with the identification by sequencing of the N-terminal 11 amino acids of gp91^{phox} in a fragment purified after endoproteinase treatment of cytochrome b_{558} [4]. The similarity in the hydropathy plots between gp91^{phox} and its homologues suggests that their N-termini would also protrude to the cytosolic face of the membrane. This information is very important and shows that gp91^{phox} possesses an even number of transmembrane helices (most likely to be 6), as the C- and N-terminal parts of the protein co-localize in the cytosol. Moreover, the protrusion of the N-terminal part of Nox2 (and its homologues) in the cytosol raises the possibility that this small region could be a docking site for proteins involved in the regulation of the NADPH oxidase activity.

The work was supported by a grant number H0604 from the Arthritis Research Campaign, U.K., by grants from the Ministère de l'Enseignement supérieur de la Recherche et Technologie, Paris, the Région Rhône Alpes, programme Mobilité Internationale Rhône Alpes 2001, délégation de Grenoble, the Fondation pour la Recherche Médicale, Isère, and from the French association 'La Ligue Nationale contre le Cancer'. We thank Helen Kennedy and Robert Meech, Department of Physiology, University of Bristol, for the use of the Bio-Rad MRC 600 Confocal Microscope, and Marie-Claire Joseph for technical assistance.

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Received 7 June 2004/30 June 2004; accepted 2 July 2004
Published as BJ Immediate Publication 2 July 2004, DOI 10.1042/BJ20040954